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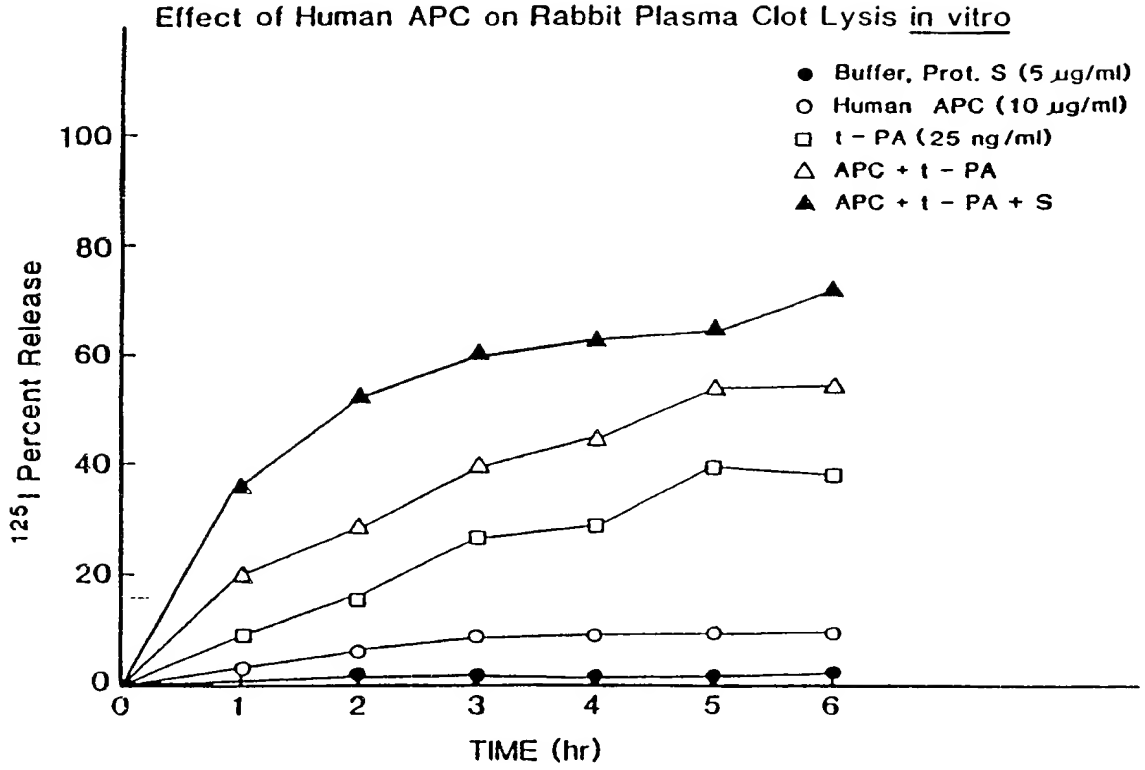
(54) Title: THERAPEUTIC THROMBOLYTIC COMPOSITION



(57) Abstract

A new therapeutic combination of t-PA and APC provides protection against blood clot reformation.

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TITLE: THERAPEUTIC THROMBOLYTIC COMPOSITION

BACKGROUND OF THE INVENTION

Technical Field:

The present invention is related generally to the field of thrombolytic agents. More specifically the present invention is related to the combination of two fibrinolytic agents, Tissue Plasminogen Activator (t-PA) and Activated Protein C (APC), to produce a unique therapeutic thrombolytic composition.

State of the Art:

Tissue Plasminogen Activator is a trace plasma protease with a molecular weight of about 72,000 which plays a central role in a number of physiological processes. Of these, its fibrinolytic activity has received the most attention. Its fibrinolytic activity is believed to involve its affinity for fibrin and its ability to form a fibrin/t-PA complex on the surface of recently-formed fibrin clots. Once formed, the fibrin/t-PA complex activates plasminogen to form plasmin, an active thrombolytic agent. To date, at least two fast-acting inhibitors of t-PA, Plasminogen Activator Inhibitor (PAI) I and II, have been identified in plasma

and have been shown to inactivate t-PA through complex formation (Sprengers and Kluft, Blood 69:38, 1987). In the presence of PAI I and II, t-PA exhibits a half-life of only two to three minutes in the circulatory system.

Like t-PA, Protein C is also a trace plasma protease zymogen. Unlike t-PA, when activated to an active protease, APC acts as a potent and specific anticoagulant whose activity has been described by many authors (Stenflo, J. Biol. Chem. 251:355, 1976; Kisiel et al, Biochem. 16:5824, 1977; Marlar et al, Blood 59:1067, 1982). The mechanism of its anticoagulant effect involves the proteolytic cleavage of Factors Va and VIIIa to form inactive enzymes. Unlike t-PA, APC's activity appears to be species specific. It has been reported that bovine APC, despite considerable amino acid sequence homology with human APC, has negligible anticoagulant activity in human plasma (Kisiel, J. Clin. Invest. 64:761, 1979; Walker Thom. Res. 22:321, 1981). Although the exact mechanism of APC's species specificity is not known, a possible explanation has been proposed by Walker supra. that the negligble anticoagulant activity is due to a lack of interaction between the bovine APC and human Protein S.

In addition to its anticoagulant activity, recently it has been demonstrated that bovine and human APC can act as a profibrinolytic agent. Several investigators have demonstrated elevated t-PA activity following infusion of APC into animals (Comp & Esmon, J. Clin. Ivest. 68:1221, 1981; Burdick and Schaub Throm. Haem. 45:413, 1987). APC's profibrinolytic activity, like its anticoagulant activity, is highly species specific (Walker supra.), but the species specificity can

apparently be overridden with concomitant use of the autologous form of Protein S, APC's cofactor (Walker, supra). Although the mechanism by which APC exerts its profibrinolytic effect is unclear, it has been shown that APC can form a complex with PAI, thereby neutralizing PAI activity (Sakata et al, Blood 86:1218, 1986). These same authors have also demonstrated that t-PA itself is able to dissolve clots in a clot lysis assay. It should be noted, however, that in these experiments the addition of APC did not change either (1) the kinetics or (2) the quantity of t-PA required for maximum clot lysis within the experimental time period (20 hours). It should be noted that the addition of PAI did significantly inhibit clot dissolution by t-PA in the absence of APC. Importantly, PAI had no effect on clot dissolution when excess APC was present along with t-PA. In the same study, it was reported that human APC was less effective than its bovine counterpart in dissolving clots in human plasma.

In yet another study, Taylor et al, (Throm. Res. 37:639, 1985) showed a dose-dependent profibrinolytic effect of human APC in an in vitro clot lysis assay. These investigators demonstrated approximately 65 percent clot lysis at 25-30 hours with human APC (20 µg/mL) in human whole blood. However, under the same conditions the authors were unable to demonstrate clot lysis in either citrated human plasma or platelet poor plasma.

Currently t-PA is undergoing clinical evaluation as a thrombolytic agent at doses approaching 100 to 150 mg per patient. Bleeding, observed in approximately 20 percent of those treated with t-PA, is the most common side effect and appears to be dose related. Consequently,

reducing the dose of t-PA administered to patients with thrombus formation is a crucial goal of this approach to thrombolytic therapy.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to combine two previously-described proteins, t-PA and APC, to produce a unique therapeutic thrombolytic composition with fibrinolytic and anticoagulant activity.

It is another object of the present invention to provide a method for inducing blood clot dissolution comprising contacting the blood clot with the composition of the present invention for sufficient time to produce thrombolytic effect and protection against blood clot reformation.

It is a further object of the present invention to reduce the dose of t-PA (recombinant or mammalian) required for effective thrombolysis and minimize the incidence of side effects thereof.

Other objects and advantages of the present invention will become evident from the Detailed Description of the Invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed

description when considered in connection with the accompanying drawings wherein:

Fig. 1 shows the results of various treatments on blood clot dissolution;

Fig. 2 shows the results of various treatments on blood clots lysis using a radiolabeled marker; and

Fig. 3 shows the effect of human APC in combination with t-PA on rabbit plasma clot lysis with or without Protein S.

DETAILED DESCRIPTION OF THE INVENTION

The above and various other objects and advantages of the invention are achieved by a thrombolytic or anticoagulant composition, comprising an admixture of tissue Plasminogen Activator (t-PA) and activated Protein C (APC), each in an amount substantially less than that required for t-PA or APC alone to produce thrombolytic or anticoagulant effect.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

In general the following methods were used. Human pooled plasma was clotted at room temperature (about 22°-25°C) by addition of of human thrombin and the resultant clot was suspended in 25mM Tris buffer containing 0.1 M NaCl and 5mM $CaCl_2$ pH 7.5. The concentrations of human APC (4.5 µg/mL) and t-PA (50 ng/mL) used for the clot lysis assay are only illustrative. After adding the test agents, all samples were incubated at 37°C. In all examples cited below, clot lysis was maximal when the combination of t-PA and APC was used. Since different diseases may respond differentially to different ratios of the two agents (t-PA and APC), it is clear that a variety of therapeutic regimens comprising different ratios of these two pharmocologic agents can be formulated to treat specific condition(s). Because t-PA is not known to be species specific, either single or 2-chain t-PA from any source could be employed. Since APC's species specificity is most probably due to its interaction with the autologous protein, Protein S, it is apparent that in place of human APC, one could substitute a combination of APC and Protein S as long as they are obtained from the same species or obtained by recombinant technology.

E X A M P L E I

In order to demonstrate that the combination of APC and t-PA exhibits unexpected synergistic or enhanced fibrinolytic activity, a simple <u>in vitro</u> experiment was designed to establish that the combination is more effective in dissolving blood clots than the individual components at the same concentration or dosage level. In this test, clots were formed in each of the four test tubes by adding 100 µL of 25 mM $CaCl_2$ and 10 µL of

thrombin (1 μM) to 0.5 mL pooled human plasma. The time required for clot dissolution was measured following the addition of 500 μL of one of the following four reagents: (1) Tris/NaCl buffer 5mM $CaCl_2$, (2) 20 μL APC (5 μg) and 25 μL t-PA (0.05 μg), (3) 20 μL APC (5 μg) and (4) 25 μL t-PA (0.05 μg). Table 1 shows the results obtained which were as follows.

Clot dissolution was minimal in the test tubes treated with Tris/NaCl 5mM $CaCl_2$ buffer or APC as a single agent (test tubes 1 and 3); clot dissolution was complete within 20 hours with single agent t-PA (test tube 4); the combination of t-PA and APC results in 80 percent dissolution at three hours with dissolution complete at four hours (test tube 2). Figure 1 shows the results obtained at three hours.

T A B L E 1

Test Tube #	Agents	Clot Dissolution (%) at 3 Hrs.
1	Buffer	<10
2	5 μg APC + 0.05 μg t-PA	80 - 90
3	Human APC (5 μg)	10 - 20
4	t-PA (0.05 μg)	40 - 50

EXAMPLE II

A second experiment was designed to more accurately quantitate the results. The test was conducted using clots formed essentially as described in Example I but which were in addition radiolabeled by incorporating about 770,000 cpm of ^{125}I-fibrinogen into each clot. As shown in Panels A, B and C of Figure 2, these clots were then treated in one of the following ways: (1) with 4.5 µg of single agent APC (open circle); (2) one of three concentrations of single agent t-PA represented by the square (Panel A - 0.0125, Panel B - 0.025 and Panel C - 0.05 µg) and (3) combinations of APC and t-PA represented by the triangles at their corresonding single agent concentrations. Clot dissolution was quantitated with a gamma counter by counting 25 µL aliquots from each test tube at hourly intervals beginning at time 0 and continuing for six hours. As shown in Figure 2, these data indicate that when APC at 4.5 µg is combined with t-PA, at 50 ng/mL, the combination yields complete clot dissolution at one-half of the t-PA concentrations (Panel B) required by single agent t-PA (Panel C) for complete lysis in the same period of time. Table 2 summarizes these data.

TABLE 2

Agents	^{125}I Released (%) at 6 Hrs.
Human APC	06
t-PA (12.5 ng/mL)	31
t-PA (25 ng/mL)	38
t-PA (50 ng/mL)	80
APC + t-PA (12.5 ng/mL)	68
APC + t-PA (25 ng/mL)	85
APC + t-PA (50 ng/mL)	88

EXAMPLE III

To further elucidate the conditions of APC's species specificity, a third in vitro test was conducted using clots formed in rabbit plasma. To form the clots, 0.1 mL of 25 mM $CaCl_2$ and 10 µL of ^{125}I-fibrinogen (about 500,000 cpm) were added to 0.5 mL of citrated rabbit plasma. The clots were initiated by the addition of 10 µL of 1 µM human thrombin. The resultant clot was suspended by addition of 0.5 mL of 25mM Tris buffer containing 0.1M NaCl and 5mM $CaCl_2$, pH 7.5. As shown in Figure 3, clot lysis was measured following the addition of (1) buffer and human Protein S (5 µg/mL); (2) single agent t-PA (25 ng/mL); (3) t-PA (25 ng/mL) in combination with human APC (10 µg/mL) and (4) t-PA (25ng/mL), human APC (10 µg/mL) and human Protein S (5 µg/mL). At hourly intervals, 25 mL aliquots of supernatant were withdrawn and counted in a gamma counter. Results, as shown in Figure 3, indicate that, t-PA in combination with human APC and its cofactor human S, produced greater than 50 to 70 percent clot lysis at 2 and 6 hours, respectively. However, the same combination in the absence of human S

produced only 20 to 50 percent clot lysis. These results clearly indicate that species specificity of APC can be overcome by using autologous Protein S (See Table 3).

TABLE 3

Agents	^{125}I Released (%) at 6 Hrs.
Buffer, Protein S (5 μg/mL)	01
Human APC (10 μg/mL)	10
T-PA (25 ng/mL)	37
10 μg APC + 25 ng/mL t-PA	52
10 μg APC + 25 ng/mL t-PA + Protein S (5 μg/mL)	70

Since, the side effect of hemorrhage encountered with t-PA at the currently recommended clinically effective dosage level of about 100-150 mg per patient, is a serious problem in its use as a thrombolytic agent, the present invention, for the first time, makes it possible to alleviate this dangerous side-effect by reducing the dosage level of t-PA while still achieving effective thrombolysis when such reduced dosage level of t-PA is combined with APC, optionally with autologous Protein S. In addition, reducing the t-PA level needed for effective treatment also reduces the cost of this type of therapy.

For the practice of the present invention, APC or t-PA can be obtained synthetically, by recombinant genetic technology, from mammalian source or by any other means or method and either the whole molecule or, a biologically active part thereof can be employed so long as substantially similar results are obtained.

It should be noted that an important additional advantage of the present invention is that the combination of APC and Protein S provides the protection against arterial reocclusion due to rethrombosis, since the combination of APC and Protein S is known to have potent anticoagulant and profibrinolytic activity. Restenosis is a major problem with patients receiving t-PA therapy for clot dissolution. Furthermore, APC ± protein S should be useful in reducing the rate of restenosis in any patient, in which this has been shown to be a problem.

The synergistic effect obtained with the therapeutic combination as demonstrated herein opens a new vista for the treatment of those conditions which result from thrombus formation. Of course, the composition of the present invention can be administered in any pharmaceutically acceptable vehicle, if necessary, such as non-toxic sterile buffer, physiological saline and the like and it can be administered through any suitable route such as orally, intramuscularly, intravenously and the like.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

WHAT IS CLAIMED IS

1. A thrombolytic or anticoagulant composition, comprising an admixture of tissue Plasminogen Activator (t-PA) and activated Protein C (APC), in which t-PA is in an amount substantially less than that required for t-PA alone to produce thrombolytic effect, said composition being effective either as a fibrinolytic or anticoagulant.

2. The composition of claim 1 further comprising autologous Protein S.

3. The composition of claim 1 wherein the APC is in an amount, at a constant t-PA concentration, which reduces the time required for thrombolysis.

4. A method for blood clot dissolution comprising contacting blood clot with an effective amount of the composition of claim 1 for sufficient time to produce thrombolytic or anticoagulant effect.

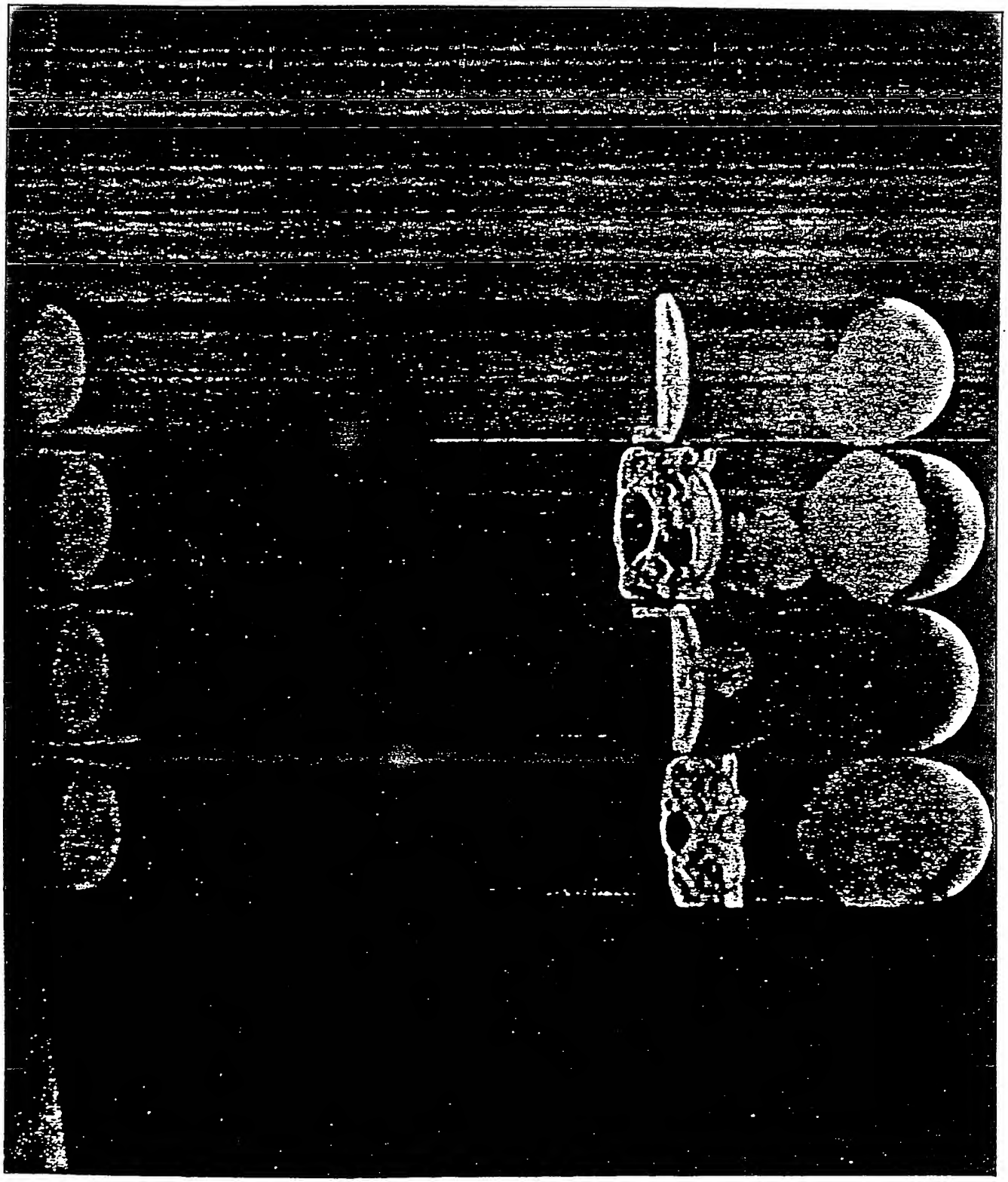


FIG. 1

FIG. 2 A

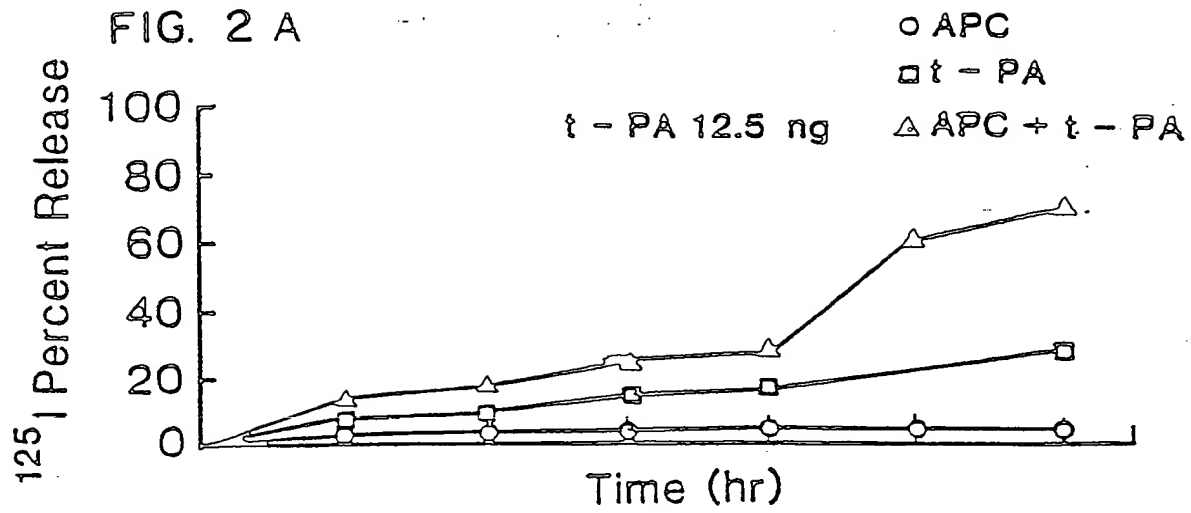


FIG. 2 B

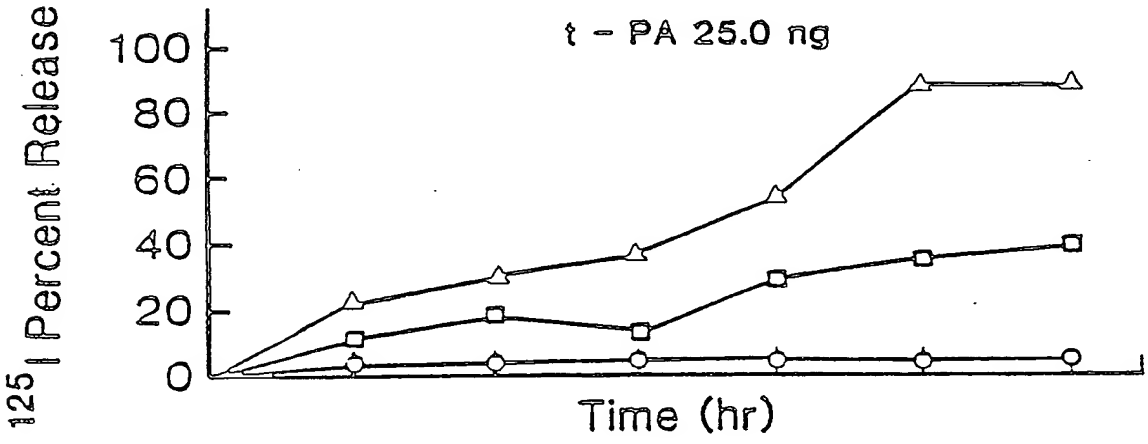


FIG. 2 C

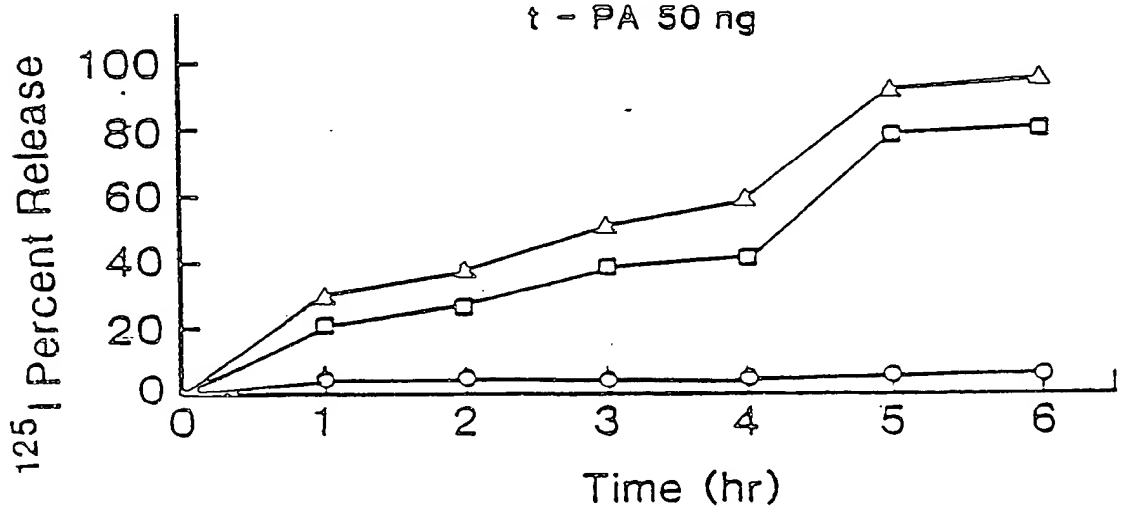
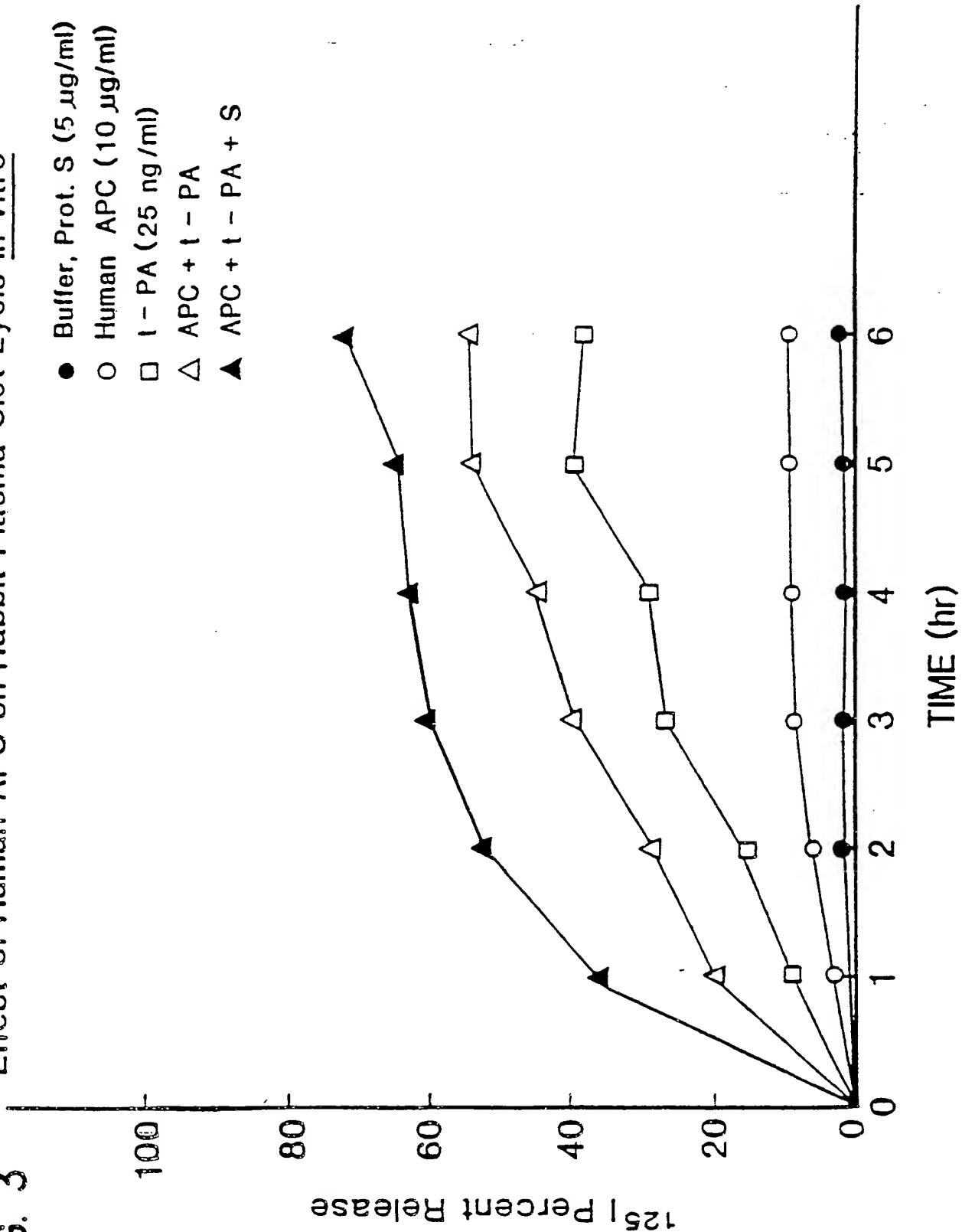


FIG. 3


Effect of Human APC on Rabbit Plasma Clot Lysis in vitro
● Buffer, Prot. S (5 µg/ml)
○ Human APC (10 µg/ml)
□ t-PA (25 ng/ml)
△ APC + t-PA
▲ APC + t-PA + S
^{125}I Percent Release
100
80
60
40
20
0
0
1
2
3
4
5
6
TIME (hr)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	COMP ET AL, "Generation of Fibrinolytic Activity by Infusion of Activated Protein C into Dogs", Journal of Clinical Investigation, issued November 1981, volume 68, 1221-1228, see the entire document.	1, 3 and 4
Y	US, A, 4,552,760, (MURAKAMI ET AL), 12 November 1985, see columns 5 and 6.	1, 3 and 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/03192

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.: 424/94.2, 94.63

IPC[4]: A61k 37/48; C12N 9/48

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/94.2, 94.64, 101; 435/212; 530/380; 514/2,21

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CASONLINE, BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	DE FOUW ET AL, "The Cofactor Role of Protein S in the Acceleration of Whole Blood Clot Lysis by Activated Protein C In Vitro", Blood, issued April 1986, volume 67, number 4, 1189-1192, see the entire document.	1-4
Y	TAYLOR ET AL, "A New Function For Activated Protein C: Activated Protein C Prevents Inhibition of Plasminogen Activators by Releasate from Mononuclear Leukocytes-Platelet Suspensions Stimulated by Phorbol Diester", Thrombosis Research, Issued 1985, volume 37, number 1, 155-164, see pages 155 and 162-163.	1, 3 and 4

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 November 1988	11 JAN 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	J. M. Stone